SPERMATOGENESIS AND RELATED PHENOMENA IN ALFALFA.

by

Alfred E. Clarke

A Thesis

Submitted to the University of Alberta in partial fulfilment of the requirements for the Degree of Master of Science.

Edmonton, Alberta.
April, 1927.

U N I V E R S I T Y O F A L B E R T A

FACULTY OF AGRICULTURE.

The undersigned hereby certify that they have read and recommend to the Committee on Graduate Studies for acceptance, a dissertion on "Spermatogenesis and Related Phenomena in Alfalfa," submitted by Alfred E. Clarke, B.A., in partial fulfilment of the requirements for the degree of Master of Science.

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Introduction.

During recent years the growing of registered alfalfa seed has become an important undertaking on the irrigated lands of southern Alberta, particularly in the Brooks district. Alfalfa seed is grown on a larger scale in the Brooks district and in southern Ontario than anywhere else in the Dominion of Canada and these two regions are recognized as being among the largest alfalfa seed-growing areas on the North American continent. The growing of registered alfalfa seed has proved profitable for the seed sells readily at a good price. Unfortunately, however, the size of the seed crop fluctuates a great deal from year to year owing to climatic and other environmental factors. Also, individual plants vary a great deal in their seed-setting capacity, that is, two plants of the same variety may be grown side by side and one of these plants may form a large number of pods while the other may form very few.

HYBRIDIZATION EXPERIMENTS AND RELATED PHENOMENA IN ALFALFA.

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Introduction.

During recent years the growing of registered alfalfa seed has become an important undertaking on the irrigated lands of southern Alberta, particularly in the Brooks district. Alfalfa seed is grown on a larger scale in the Brooks district and in southern Ontario than anywhere else in the Dominion of Canada and these two regions are recognized as being among the largest alfalfa seed-growing areas on the North American continent. The growing of registered alfalfa seed has proved profitable for the seed sells readily at a good price. Unfortunately, however, the size of the seed crop fluctuates a great deal from year to year owing to climatic and other environmental factors. Also, individual plants vary a great deal in their seed-setting capacity, that is, two plants of the same variety may be grown side by side and one of these plants may form a large number of pods while the other may form very few.

Since it is important that knowledge regarding the factors which influence seed-setting in alfalfa should be increased, a study of this problem was commenced at the University of Alberta in the spring of 1935. Previous work done elsewhere on the seed-setting problem has been confined almost entirely to field observation and superficial field experiments. By combining a study in the field with experimental work in the greenhouse and the laboratory, paying particular attention to the normal behavior during the processes of spermatogenesis, oogenesis, fertilization and embryogeny, and studying any abnormalities which might be found during these stages, it was hoped that data might be secured regarding the fundamental basis of the problem. Obviously, an exhaustive study of all aspects of the seed-setting problem would necessitate long and intensive research. Consequently, the present investigation, which has been carried out at the suggestion and under the direction of Professor J. R. Fryer, has been confined to a study of spermatogenesis and related phenomena in alfalfa.

Review of the literature.

Probably the most important microscopic work which has been carried out with alfalfa is a study made by Martin (40) of the development of the ovule in five species of legumes, including Medicago sativa. Castetter (13) has studied pollen development in Mel-

ilotus alba and Miss Latter (37) has made similar studies with Lathyrus odoratus. Weinstein (51) has made cytological studies on the development of both the anther and ovary of Phaseolus vulgaris. Literature dealing with spermatogenesis in alfalfa is negligible. Elders (26) has reported the chromosome number for Medicago sativa as being thirty-two in the diploid condition. All of his counts, however, were obtained from root tips and he made no preparations of the anther.

While no microscopic studies have been made on pollen development in alfalfa, some investigations have been carried out which treat of the seed-setting problem in relation to environmental factors.

Moisture conditions are known to have an important effect. Stewart (50) states that all the large alfalfa seed-producing zones are in arid or semi-arid regions. When an abundant supply of soil moisture is present, alfalfa grows very rapidly and gives a high hay yield but sets little seed. During the growing of the seed crop it seems necessary that a constant supply of moisture be maintained somewhere within reach of the plant, but at no period should it be so easily available as to induce rapid vegetative growth. Blinn (7) in 1919 found at the Colorado Experimental Station that alfalfa seed failed to set under field conditions due, it was thought, to abnormally wet weather during the spring and sum-

mer months, yet plants grown in cement pots which received heavy applications of water all set seed fairly well. When water in varying amounts was applied to plants grown in cement pots, the results did not show that alfalfa seed yields could be controlled by the application of any definite amount of water. This would seem to indicate that there are other factors influencing seed-setting besides any particular moisture supply.

Martin (41) found that there are two causes of failure of seed to set in alfalfa,-the failure of the pollen to germinate and the "blasting" of the seed. The germination of the pollen depends upon a proper supply of moisture. The water requirements for the germination of the pollen depends upon a certain ratio between the moisture delivered by the stigma and the moisture of the air surrounding the stigma. The "blasting" of seed is due to the arrested development of the embryo owing either to the plant's inability to furnish the proper water and food supply for the maturing of the seed during drought or to some pathological conditions to which the seed is more susceptible under drought conditions.

Heat and light also seem to be important climatic factors. Piper and his co-workers (42) have shown that hot sunshine induces automatic tripping, that is, tripping without the interference of other natural agencies such as insects. Piper also showed

that tripping is necessary for pollination. Aicher (1) believes that there is a close correlation between the number of days of sunshine during the summer season and the amount of seed-setting. Blinn (7) found that plants growing along the edge of a dry bare ditch set seed heavily on the branches nearest the ground, while the balance of the branches were practically barren of seed. This, he thought, was due to the dry, sandy surface reflecting both heat and light.

Gray (30), working at Lethbridge, Alberta, found that the wind is a very poor factor in tripping alfalfa blossoms and is of value in only the most unusual cases.

Pollination may be effected by long-tongued insects such as leaf-cutter bees (Megachile) and bumblebees or by automatic tripping. Pollination by insects brings about cross-fertilization while automatic tripping brings about self-fertilization. In the opinion of Professor Strickland of the Department of Entomology, University of Alberta, the leaf-cutter bee is very common throughout Alberta.

Piper (42) found that bright sunshine induces automatic tripping. Owing to the latter phenomenon it is possible to obtain good seed yields in regions where insects adapted for tripping are not numerous. According to Stewart (50) alternating spells of cloudy and bright weather with moderate wind and occasional

showers are considered favorable for automatic tripping.

Hay (31) has studied the effect of artificial tripping on seed-setting at Lethbridge, Alberta. He found that 9.48 per cent. of the artificially tripped flowers and 5.94 per cent. of the flowers used as checks set seed and concluded that with such a low percentage of seed pods from the flowers that were tripped, evn though it was almost double that from the checks, lack of tripping was not the limiting factor. He found that neither the time of day when the tripping was performed nor the color of blossoms showed any relation to seed-setting. Since he tripped only 960 flowers, using the same number as a check, the investigation was not performed on a large enough scale to be conclusive. In some unpublished work done at the University of Alberta by the writer it was found that a very much higher percentage of seed than that reported by Hay was obtained. However, the plants at Edmonton were spaced three feet each way while at Lethbridge they were grown in rows, and plants which are thinly spaced always tend to set seed more freely.

Seamans (45) found that alfalfa thrips, especially Frankliniella occidentalis Pergande, are very prevalent in Alberta, and that seed production in alfalfa is materially reduced by these insects in two ways. The most severe losses are caused by the thrips feeding on unopened buds, while lesser

losses are caused by the thrips feeding on the ovaries of unopened flowers or on the young seed pods. At Lethbridge, in 1922, caged blossoms which were tripped artificially, but which contained thrips, were so badly injured that only sixty per cent. of the flowers set seed with an average of 3.4 seeds per pod, while the thrips-free blossoms under the same conditions resulted in a one hundred per cent. seed setting with an average of six seeds per pod.

Materials and methods.

Twenty-three varieties and strains, as shown in Table I, were available for study.

All of these strains were grown in the field, in rows three feet apart, the individual plants being spaced three feet apart within each row, Plants were also grown in the greenhouse. Some of the latter were halved and the halves grown under different moisture and temperature conditions. In this way the effect of environment could be measured without being complicated by inherent differences between different plants.

Also an abnormal plant was found in a field of Arctic sweet clover planted from seed obtained from the University of Saskatchewan. Professor Kirk has suggested that such aberrant plants are natural hybrids between sweet clover and alfalfa.

Temporary mounts with fresh material were made by Belling's smear method, anthers being teased out

TABLE I

Species.	Variety or strain.	Source of seed and origin of stock.
M. media	Grimm- "Grafton" strain	Mass selection from superior plants of "Wiley" stock, made by Mr. Grafton, Brooks, Alberta.
M. media	Grimm- "Wiley" strain	C. P.R. Farm, Brooks. Stock seed produced by H. K. Wiley, Springfield, Idaho, from stock originally secured from A. B. Lyman, Excelsior, Minnesota.
M.media	Grimm- "Kirk" strain.	University of Saskatchewan.
M. media	Grimm- "1917 seed"	Saskatchewan.
M. media	Grimm- "Lyman" strain	Bassano Farm, C. P. R. Irrigation Investigation Branch from seed obtained from A. B. Lyman of Excelsior, Minnesota.
M. media	Grimm- "Daniel" strain	C. P. R. Brooks, Alberta. Stock seed from O. L. Daniel of Millicent, Alberta, who previously obtained his stock from Dr. W. M. Williams of Harlem, Montana.
M. media	Grimm- "Williams" strain	C. P. R. Farm, Brooks. St ock seed grown by Dr. W. M. Williams of Harlem, Montana. Dr. Williams originally obtained his alfalfa from Lyman.
M. media	Cossack	University of Saskatchewan.
M. media	Hardy Michigan	Macdonald College.

TABLE I (Continued)

Species.	Variety or strain.	Source of seed and origin of stock;
M. media	Liscomb	Wheatlands, Ltd., Saskatchewan.
M. media	Cherno	University of Saskatchewan.
M. media	Baltic	Wheatlands, Ltd., Saskatchewan.
M. media	Sand Lucerne	Saskatchewan. Originally from S. P. I. No. 23394.
M. sativa	Turkestan	Saskatchewan. Originally from S. P. I. NO. 21032.
M. sativa	"Argentine"	Tucuman, Argentine. S. P. I. No. 56853.
M. media	Hansen's Hybrid No. 1	
M. media	"Southworth's Hybrid"	Seed from F_1 generation, the flowers of which were selfed and exposed, of an alfalfa X falcata cross made by Professor Southworth of Manitoba Agricultural College.
M. media	Grimm's Centurion	Macdonald College
M. falcata	Obb	University of Saskatchewan.
M. media	North Sweden	Macdonald College.
M. falcata	"Russia"	Russia. S. P. I. No. 42741.
M. media	Ontario Variegated	Ontario Agricultural College.
M. media	"Disco 19A"	Brooks, Alberta. Original stock obtained from Dakota Improved Seed Company, Mitchell, South Dakota.

in the stain with nickel instruments and the cover glass being sealed with gum damar or vaseline. Later soft melted paraffine was used for sealing and gave better results. Owing to difficulty in obtaining fresh material from the greenhouse during the earlier part of the winter months some of the smears were made from material previously fixed in formalin-acetic-alcohol.

For permanent mounts prepared by the paraffine method, flowers were fixed at various stages from the very smallest buds until after fertilization. Several fixing agents were employed. Formalin-acetic-alcohol was used in the following proportions:

Glacial acetic alcohol..... 5 c. c.
Commercial formalin..... 5 c. c.
70% alcohol.....90 c. c.

The advantage of this fixing agent is that the material may be left in the solution for months.

Carnoy's fluid was also used for some of the earlier fixations. It was made up as follows:

Absolute alcohol..... 6 parts
Chloroform..... 3 parts
Glacial acetic alcohol..... 1 part

Excellent results were obtained from the use of Bouin's fixing agent as modified by Allen, the composition of which is given below:

Picric acid, saturated aqueous solution... 75 c. c.

Commercial formalin..... 25 c. c.

Glacial acetic acid.....; 5 c. c.

Urea crystals..... 2 gm.

Chromic acid crystals..... 1.5 gm.

The material was fixed in this solution for several hours after the air had been evacuated from the tissues by means of an air pump. After fixing the material was run from water up to 75 per cent. alcohol in one hour, and thoroughly washed in 75 per cent. alcohol to which a few drops of lithium carbonate had been added. The material was then dehydrated and embedded in paraffine in the usual way.

Preparations were sectioned at a thickness of about 10 u.

Among the stains used were aqueous safranin and the quick haematoxylin method described by Cole (26). Satisfactory results were obtained by using aqueous Delafield's haematoxylin, the best differentiation being secured when the sections were overstained and then partially destained in 70 per cent. alcohol to which a couple of drops of hydrochloric acid had been added.

Examinations were also made of mature pollen stained with chloral-hydrate-iodine solution (water 50 c.c.,chloral hydrate 50 gm.,iodine 1 gm., and absolute alcohol 50 c.c.) as described by Longley.(38). Pollen grains were also stained by Bel-

ling's iron-acetocarmine stain. To determine the percentage of sterile pollen present, flowers were tripped, the anthers removed by means of fine forceps, and the anther sacs teased apart in a drop of Longley's solution placed upon a slide. The remains of the anther were then removed and a cover glass placed over the pollen grains left in the solution. By microscopic examination it was possible to distinguish the empty pollen grains from those possessing protoplasmic contents. In order to make conditions uniform, only flowers were used which were ready for tripping but had not yet tripped.

Pollen was also germinated in sucrose solutions of varying strengths, the best results being obtained with thirty per cent. solutions. However, sucrose solutions did not prove satisfactory because the pollen tubes did not attain any appreciable length before bursting. Better results were obtained by germinating the pollen on a 1½ per cent. agar medium containing about 25 per cent. sucrose. The germinated grains were transferred to a slide, stained and mounted, the cover glass being sealed with paraffine. Methyl green in glycerine proved a satisfactory stain. The nuclei could be seen to advantage when a Wratten F photomicrographic filter No. 29 was used.

Development of the stamens.

In the alfalfa blossom there are ten stamens. The stamens arise in two whorls. In the young bud there is a whorl of long stamens and a whorl of short ones, a long and a short stamen alternating. (Fig.1) The two whorls, however, originate at the same level but the long stamens are older than the short ones and are always found to be at a slightly more advanced stage of development. Consequently when a young bud is sectioned anthers will be found at two stages of development. Nine of the ten stamens become joined together at their base to form a stamen sheath or tube. The tenth stamen, the posterior one, remains free. This stamen never grows to the same length as the remaining nine. At maturity the difference in length between the two sets of stamens is not so noticeable as in the earlier stages.

Development of the wall layers of the anther.

The development of the anther is, in general, similar to the usual method of anther development found in the majority of angiosperms. Like most species, the anther produces four microsporangia. (Fig. 2) However, four archesporial regions are not found in all legumes for Miss Frith (27) has reported that the anther of Trifolium repens is bilocular, that is, it possesses only two anther lobes and

only two microsporangia are formed.

The microsporangium of alfalfa becomes differentiated off at a very early stage. When it can first be definitely distinguished it consists of a hypodermal column possessing in cross-section from three to five cells. On the outside of the young anther is a single layer of epidermal cells. Just within it is a single hypodermal layer. The cells of this initial hypodermal layer divide to form the primary parietal layer to the outside and the primary sporogenous layer to the inside. The first division of the primary parietal cells gives rise to two layers, the inner becoming the tapetum, the outer dividing a second time to form the endothecium adjoining the epidermis and the so-called "middle layer" between the endothecium and the tapetum. Consequently three layers are derived from the primary parietal cells,-the endothecium, the "middle layer" and the tapetum. (Fig.3) These conclusions regarding the development of the outer layers of the microsporangium have been deduced from the appearance and position of the cells in question as the actual cell divisions were not observed.

The cells of the "middle layer" are long, narrow and rather inconspicuous. In no instance was this layer found to be more than a single cell thick. Indeed, in some places it may be missing.

The tapetum is differentiated off at an early stage and can be readily distinguished by the time the spore mother cells are formed. Throughout their life-history the tapetal cells remain uninucleate. These tapetal cells are quite large. They commence to break down at about the tetrad stage of pollen development. By the time the pollen grains are rounded off these cells are breaking down rapidly (Fig.4), and by the time the anther has reached maturity the "middle layer" and the tapetal layer have completely disappeared.(Fig.5)

The endothecial cells increase in size until they form the chief part of the anther wall. (Fig. 5) The epidermal cells remain as a broken layer of irregularly shaped cells on the outside.

In a transverse section through a mature anther, the tissue separating the two adjacent microsporangia is found to have broken down. The line of dehiscence is located at this point (Fig. 6). Specialized stomial cells were not observed.

The resting spore mother cell.

The primary sporogenous layer undergoes several divisions to form the spore mother cells. The mature resting spore mother cells are polygonal in outline and closely packed together. All the tissues of the young anther show no space between adjacent

cells unless, perhaps, there is sometimes a gap between the tapetal cells and the spore mother cells adjacent to the tapetum. A number of preparations show some space at this point but this may be due to slight contraction of the spore mother cells caused by the fixing agent.

The resting spore mother cell is filled with cytoplasm and possesses a very large, conspicuous nucleus, generally near the centre of the cell. This resting nucleus presents a faintly staining reticulum of granular appearance, and a large deep-staining nucleolus more or less spherical in shape and centrally located. Immediately around the nucleolus is a comparatively light-staining zone, the karyoplasm or nucleoplasm evidently being less dense in this central area than it is at the periphery of the nucleus (Fig. 7). Occasionally two nucleoli are found within a single nucleus, but no instance of the occurrence of more than two was observed. When two individuals are present each is smaller in size than when only a single nucleolus occurs. In suitable preparations, rather faintly stained, the nucleolus was found not to be homogeneous throughout but to possess vacuolated areas containing small granules called "nucleolini", "argentophile granules" or "crystal bodies" by different workers who have found them in other species. While the number of

these bodies present in a single nucleolus varies, the presence of at least one seems to be a constant feature at this stage. When more than one is found usually one is considerably larger than the others and circular in outline. (Fig. 7). Probably the endonucleolus or nucleolar body is derived from this large round body. Miss Latter (37) has suggested that these granules possibly represent depositions of protein material to be utilized by the spore mother cell during the stage of activity upon which it is about to enter. She attempted to verify her hypothesis by means of microchemical tests but without much success due to the exceedingly small size of the bodies with which she was working. During the present investigation nuclei of various somatic tissues were studied to find out if similar bodies existed in them but no conclusions could be drawn. In the tapetal cells, however, these nucleolini are present.

First meiotic division.

The beginning of the prophase of the first reduction division is marked by the contraction of the karyoplasm away from the nuclear membrane and its collection about the nucleolus in the centre of the nucleus, leaving a clear zone to the outside. (Fig. 8). The large nucleolus gradually leaves its central position until it comes in contact with the

nuclear membrane. It then becomes flattened along the membrane and its former spherical shape is lost. (Fig. 9). The karyoplasm is carried along with the nucleolus and now forms a dense knot beside it, called the synizetic knot, and the stage is known as synizesis. When more than one nucleolus is present there appears to be no relation between the directions of movement of the two nucleoli. During these early stages of the prophase thin threads begin to appear in the granular mass of karyoplasm. Miss Latter (37) has suggested that while the granular mass appears to be formed by rearrangement of the granules in linear series it is more probably due to the deposition of a stainable substance on a formerly unstainable linin thread on which the granules are situated. Judging from the large number of preparations in which synizesis was found, this stage lasts for a comparatively long time.

The synizetic knot begins to leave its position beside the nucleolus and travels towards the opposite side of the nucleus, but remains attached to the nucleolus by a few fine threads (Fig. 10). The synizetic knot continues to become more thread-like in appearance, loosening up into a long, continuous, irregularly looped thread called the spireme. This thread eventually fills practically the whole of the interior of the nucleus. This is known as the open spireme stage. Small beads of darkly

staining substance ~~substance~~ are distributed at irregular intervals along the length of the thread. Longitudinal splitting of the thread was not observed. Throughout this process, the thread remains attached to the nucleolus at a particular spot called the nucleolar body or endonucleolus. As already mentioned this body is probably derived from the large crystal body observed in the nucleolus of the resting spore mother cell. The nucleolar body is more darkly staining than the remainder of the nucleolus. The nuclear area has now attained its maximum size and there are indications that the cells are beginning to loose their polygonal outline and to round off.

The threads next thicken considerably (Fig. 12) and finally break up into the chromosome lengths. (Figs. 13 and 14). No pronounced second contraction was observed. Miss Latter (37) found that in Lathyrus odoratus during the brochonema stage the thread arranges itself into seven loops, each loop corresponding to a bivalent chromosome. In the sweet pea the haploid chromosome number is seven, but in alfalfa the haploid chromosome number is sixteen, and it would be very difficult to distinguish such a large number of loops in what is really a small nucleus. It is quite possible, however, that the procedure is similar to that described by Miss Latter. Unfortunately good preparations of the later

prophase stages were not secured and it is consequently not possible at this time to give the details of the chromosome behavior. At the close of diakinesis the nuclear membrane and the nucleolus disappear and the spindle is formed.

It was not found possible to determine whether the union of the chromosome pairs is telosynaptic or parasynaptic.

The bivalent chromosomes now become grouped on the equatorial plate of the spindle (Fig. 15). Each chromosome appears to become attached to one of the spindle fibers. The nuclear area is now scarcely distinguishable from the surrounding cytoplasm.

The chromosome pairs next separate, one member of the pair going to one pole and the other going to the opposite pole (Fig. 17). It is during the anaphase in cells showing polar views that the most satisfactory chromosome counts can be obtained. (Fig. 16). A few counts were obtained in both Medicago sativa and Medicago media. In both species the haploid chromosome number was found to be sixteen. This corresponds satisfactorily with the diploid chromosome number of thirty-two in Medicago sativa previously reported by Nilders (26). Chromosome counts were not obtained for Medicago falcata. Since Medicago media is generally accepted as a natural hybrid between the other two species, it would seem

only reasonable to suppose that Medicago falcata has the same chromosome number.

The chromosome number is of some importance in relation to sterility in alfalfa because, if Medicago falcata and Medicago sativa had different chromosome numbers, sterility in varieties of Medicago media, such as Grimm and Ontario Variegated, might be due in part to abnormal chromosome behavior resulting from such a cross. It would seem, however, that chromosome abnormalities of this sort are not a factor.

The chromosomes fuse together at the two opposite poles of the spindle in the telophase or final stage of the first division. (Fig. 18). No cell wall is formed between the two nuclei.

Interkinesis.

In the interval between the first and second divisions it would seem that the resting daughter nuclei are not completely formed, for, while the nuclear membranes are developed, no nucleoli were observed. (Fig. 19). Castetter (13) states that in Melilotus alba complete resting nuclei are constructed during interkinesis. While no discussion of the point was found in the literature, it would seem but reasonable to expect that the duration of interkinesis would depend very largely on the growing conditions.

If conditions are favorable, interkinesis would naturally be brief and a complete resting nucleus would not be constructed, perhaps even a nuclear membrane being lacking. Under unfavorable conditions, however, a longer period would elapse between the first and second divisions and consequently perhaps a complete resting nucleus would be obtained.

Second meiotic division.

Two spindles are formed simultaneously. They vary considerably in their space relationships as they may lie in the same plane (Fig. 20) or at an angle to one another.

When the telophase is reached the chromosomes fuse, the nuclear area increases in size and a nuclear membrane is formed. Finally a nucleolus is formed. In the late telophase stage the four nuclei resulting from this second division appear to have connecting strands between them (Fig. 21) but these later disappear. (Fig. 22). A tetrahedral arrangement of these four nuclei has generally been found to occur.

Origin of the spore walls of the tetrad.

No indications of wall formation are found until both the meiotic divisions are completed, So far as has been determined the walls of the tetrad are formed by the ordinary furrow method. Constrict-

ion furrows appear at the periphery and grow inwards until they meet at the centre. In this way the four spores are formed simultaneously. (Fig.23).

Development of the pollen grain.

The spores within each tetrad round off and finally separate from one another, each spore forming a pollen grain.

The wall of the mature pollen grain consists of two layers called the exine and intine. The exine is several times as thick as the intine. The intine is very thin and rather elastic. Upon the surface of the pollen grain appear three thin spots. (Fig. 25). These are probably due to the absence of the exine although it may be that the exine is present but very much thinner than in the other parts of the wall. It is through one of these pores that the pollen tube develops. Sometimes the intine begins to protrude through each pore but only a single pollen tube develops.

When the pollen grain is first formed only a single nucleus is present. This nucleus is generally centrally located. Later it divides to form the vegetative and generative nuclei. (Fig. 26). In no instance were more than two nuclei found in a single pollen grain.

Germination of the pollen grain.

By using a nutrient agar medium the alfalfa pollen grains are readily germinated. The pollen tubes frequently attain considerable length, often reaching twenty or more times the length of the diameter of the grain. Growth is quite rapid as most of it is made during the first few hours. The intine often protrudes for a considerable distance through one of the pores before bursting (Fig. 24). After germination the vegetative and generative nuclei are conveyed into the pollen tube. (Fig. 27). It would seem that the generative nucleus does not divide into the two male gametes until after the generative nucleus has been carried into the pollen tube but this division has not been observed. The pollen tube is considerably thicker at its tip than elsewhere.

Time relations of anther development.

The anther develops much earlier than the ovary. Newly formed pollen grains have been found in very small buds in which the integuments are just beginning to bud off at the base of the ovules. Pollen grains are matured very much earlier than the ovules. Coffman (19) has found that the alfalfa bud begins to shed its pollen when not more than about seven millimetres in length,

Pollen sterility.

It was found that pollen grains possessing no protoplasmic contents, and which are therefore undoubtedly sterile, are of very frequent occurrence. (Fig. 28). Consequently pollen from a large number of flowers from different plants was examined. In order to keep conditions as uniform as possible counts of the number of fertile and sterile grains were in all cases made from flowers which were ready for tripping but had not yet tripped. Grains possessing protoplasmic comtents were counted as being fertile, an assumption which may or møay not be true. It should be borne in mind, therefore, that the minimum percentage rather than the total percentage of sterile pollen was determined by this method. The results of the pollen counts are given in Table II.

From the data presented in Table II it will be seen that, within rather wide limits, the percentage of empty pollen grains found in different flowers of the same plant is constant. Wide variations are frequently found, however, between different plants, even though they belong to the same variety or strain. Counts made several days apart from different flowers belonging to the same plant showed no significant fluctuation in the percentage of sterile pollen. Also there seemed to be no correl-

Table II.

Sterile pollen grain counts.

Variety	Plant No.	No. sterile pollen grains	No. fertile pollen grains	% sterile pollen
Grafton	10.1	34	195	15
Grafton	10.1	67	423	14
Grafton	20.1	30	561	5
Grafton	20.1	9	282	3
Kirk	42.1	126	607	17
Kirk	42.1	101	407	20
Kirk	42.1	44	234	16
Kirk	43.1	118	359	25
Kirk	43.1	68	120	36
Kirk	43.1	101	321	24
Kirk	46.1	37	226	16
Wiley	33.1	107	203	35
Wiley	33.1	143	323	31
Wiley	35.1	137	374	27
Wiley	35.1	87	208	29
Lyman	69.1	11	297	4
Lyman	69.1	25	298	8
Lyman	69.1	10	154	6
Lyman	69.1	36	349	9
Lyman	69.1	46	521	8
Lyman	69.1	23	323	7
Lyman	70.1	10	292	3
Lyman	70.1	22	288	7
Lyman	70.1	8	206	4
Lyman	70.1	13	415	3
Lyman	71.1	12	252	5
Hansen's Hybrid	87.3	34	246	12
	87.3	26	193	12
Hansen's Hybrid	91.1	41	256	14
	91.1	44	255	15

Table II(Continued)

Sterile pollen grain counts.

Variety	Plant No.	No. sterile pollen grains	No. fertile pollen grains	% sterile pollen
Hansen's	----	351	107	74
Hybrid	----	339	171	66
Obb	202.1	213	247	46
Obb	202.1	201	189	52
Obb	202.1	61	108	36
Obb	206.1	75	573	12
Obb	206.1	15	152	9
Obb	206.1	45	363	12
Obb	207.1	26	225	10
Wiley-Grown	3A	78	701	10
in the green-	3A	41	315	12
house under	3A	20	316	6
hot, moist	3A	15	192	7
conditions	3A			
Wiley-Grown	3B	25	354	7
in the green-	3B	19	333	5
house under	3B	69	397	15
cool, moist	3B	53	390	12
conditions				

Each of the above counts was made from a separate flower.

ation between the amount of sterile pollen possessed by a plant and the quantity of seed set. Counts obtained from the two halves of a single initial plant grown in the greenhouse under different temperature conditions did not indicate that temperature differences alter the sterile pollen content.

As the amount of sterile pollen possessed by any given plant seems to be constant, and as the percentage is frequently very high, it may well be that this seemingly inherent difference will account in a very large measure for the wide fluctuations in seed-setting capacity exhibited by different plants of the same strain. If so, this will be an important consideration to the plant breeder. However, much more data is required before any definite conclusions in this regard can be drawn.

Not only were sterile pollen grains found but sterile tetrads were also observed.

Sterility in an aberrant sweet clover plant.

In the aberrant sweet clover plant already described a high percentage of sterile pollen was found. If, as has been suggested, this plant is a natural hybrid between alfalfa and sweet clover, a high degree of sterility is only to be expected since the haploid chromosome number for sweet clover is eight while for alfalfa it is sixteen. Kirk (36) has found considerable sterility in these aberrant plants but claims to have obtained selected lines

from the original aberrant forms which are highly fertile.

Acknowledgements.

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Summary.

1. The stamens of alfalfa develop in two whorls, one whorl being longer and more advanced than the other during any particular stage of development.
2. The anthers are, in general, similar to those found in the majority of angiosperms. Each anther possesses four locules.
3. The wall of each pollen sac consists of an epidermis, endothecium, "middle layer", and tapetum.

The "middle layer" is only one cell thick. The tapetal cells remain uninucleate throughout their life-history. As the anther matures the "middle layer" and tapetum disappear and the endothecial cells increase in size.

4. The resting spore mother cell is polygonal in outline. It possesses a large nucleus which contains a nucleolus centrally located. Within the nucleolus are found nucleolini.

5. The stages occurring in the first meiotic division are described.

6. The haploid chromosome number for both Medicago sativa and Medicago media is sixteen.

7. During interkinesis there is formed a nuclear membrane but not a nucleolus.

8. The arrangement of the spores at the close of the second meiotic division is tetrahedral. Walls are not formed until the close of the second division.

9. The spore walls are formed by furrowing.

10. The mature pollen grain has a wall composed of two layers, the intine and the exine. Three "thin spots" occur in the wall.

11. When first formed the pollen grain has only a single nucleus but this later divides to form the generative and the vegetative nuclei.

12. Pollen grains germinated on a nutrient agar medium develop pollen tubes of considerable length.

length. The pollen tube emerges through one of the "thin spots". The division of the generative nucleus into the two male gametes probably does not take place until the generative nucleus has migrated to the pollen tube.

13. Sterile pollen grains and sterile tetrads were observed. The amount of sterile pollen appears to be fairly constant for all flowers of a particular plant but wide variations in this respect were found among different plants.

14. Sterile pollen was found in an aberrant sweet clover plant.

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Explanation of plates.

Fig.1 An early stage in stamen development, showing the long and short stamens alternating. (X 80)

Fig.2 Transverse section through half of a young anther, showing the microsporangia. The tapetal cells have been shaded. (X 360)

Fig.3 Transverse section through the wall layers of an early stage of a microsporangium, showing the epidermis, the endothecium, the "middle layer", the tapetum and the spore mother cells. (X 1250)

Fig.4 Transverse section through part of a pollen sac showing the breaking down of the tapetal cells (shaded). The epidermis and the endothecium are shown. The pollen grains have just rounded off and each possesses only a single large nucleus. (X 1650)

Fig.5 Transverse section through part of the wall of a pollen sac showing the size of the epidermal and endothecial cells in comparison with a pollen grain. (X 1250)

Fig.6 Transverse section through part of a mature anther. The tissue separating two of the microsporangia has broken down. The point of dehiscence is shown. (X 340)

Fig.7 A resting spore mother cell. The nucleolus is situated in the faintly stained central portion of the nucleus. Within the nucleolus

are three nucleolini. (X 2000)

Fig. 8 Very early prophase of the first meiotic division, showing the karyoplasm contracted away from the nuclear membrane. (X 3000)

Fig. 9 The nucleolus is flattened along the nuclear membrane. (X 3000)

Fig.10 Synizesis. The synizetic knot is attached to the endonucleolus by a few fine threads. (X 3000)

Fig.11 Open spireme. (X 3000)

Fig.12 Later prophase after the thread has thickened considerably. (X 3300)

Fig.13 The thread is breaking into the chromosome lengths. (X 3300)

Fig.14 A little later stage than Fig.13. The thread has broken up still more. The nuclear membrane is not well marked and the nucleolus is becoming vacuolate. (X 3300)

Fig.15 Metaphase of the first meiotic division. (X 3300)

Fig.16 Polar view showing sixteen chromosomes. (X 3200)

Fig.17 Anaphase of the first meiotic division. (X 3200)

Fig.18 Telophase of the first meiotic division. (X 3200)

Fig.19 Interkinesis. (X 2750)

Fig.20 Metaphase of the second meiotic division

showing the two spindles lying in the same plane (X 2750)

Fig.21 Telophase of the second meiotic division, showing the connecting strands between the nuclei. (X 2750)

Fig.22 Later stage than Fig.21. The connecting strands are no longer present. Each nucleus possesses a nuclear membrane, and nucleoli are being formed. (X 2750)

Fig.23 Tetrad of spores, showing the completely formed walls. Each spore has a large nucleus in which a nucleolus is to be seen. (X 2150)

Fig.24 Germinated pollen grain, showing the intine stretched out for some distance along the pollen tube. (X 560)

Fig.25 Pollen grain with only a single nucleus. The intine is protruding through the three "thin spots". (X 4000)

Fig.26 Mature pollen grain showing the vegetative and generative nuclei. (X 1575)

Fig.27 Germinated pollen grain showing the two nuclei in the pollen tube. (X 590)

Fig.28 Photomicrograph showing fertile and sterile pollen grains within the pollen sac. (X 248)

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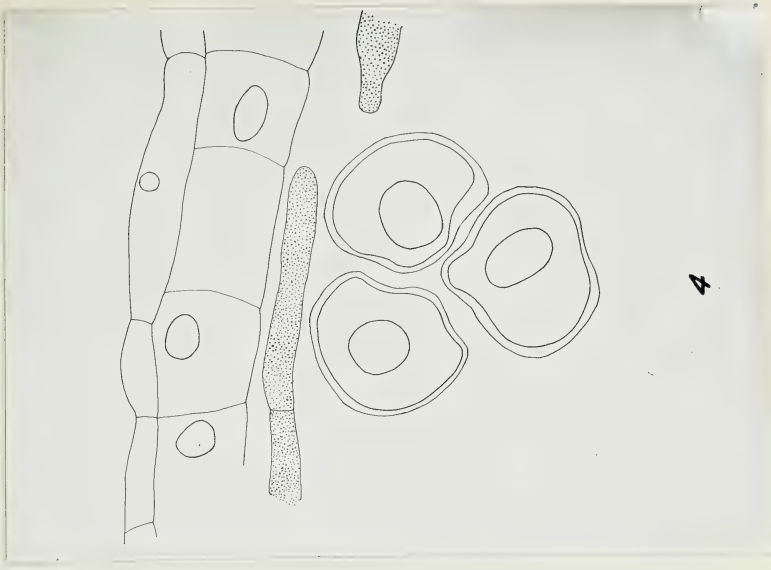

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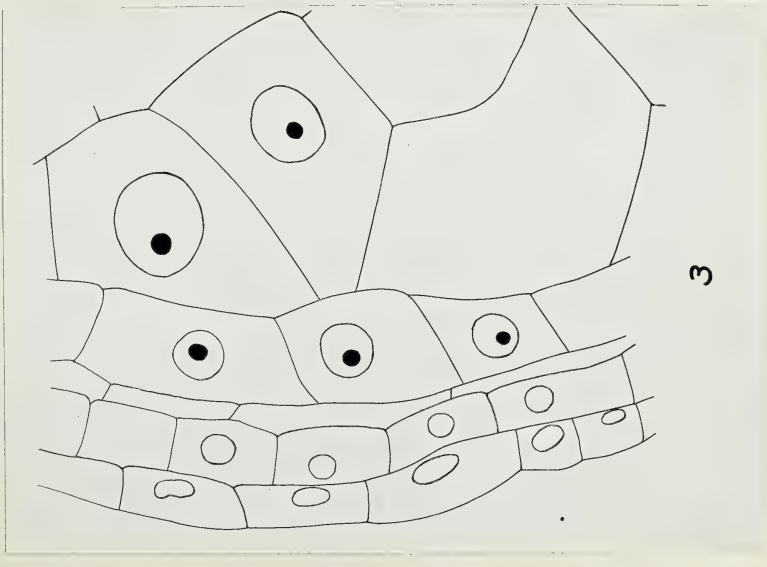

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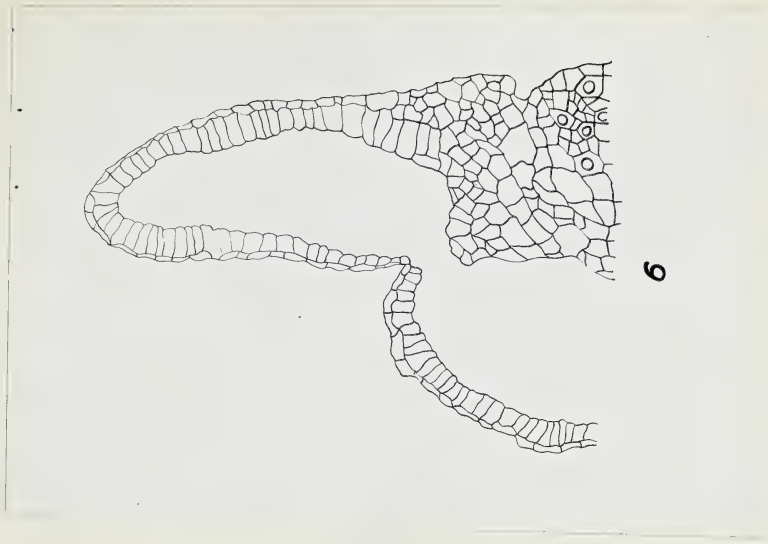

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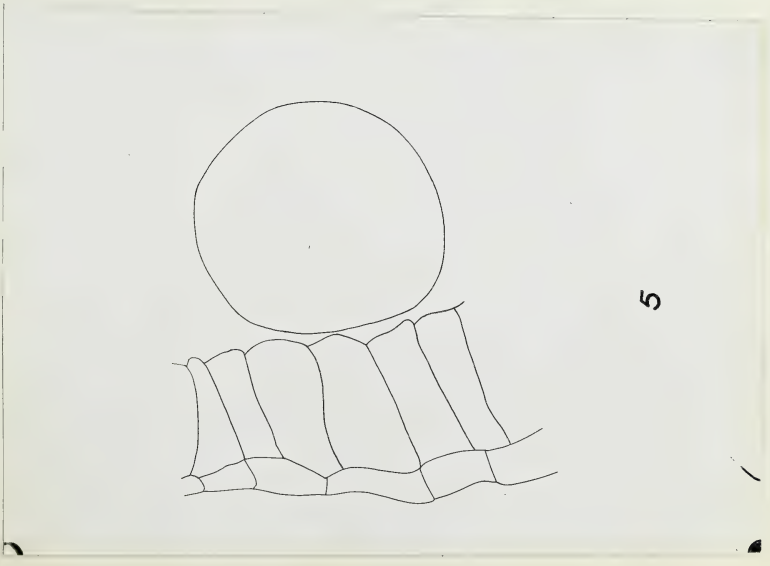

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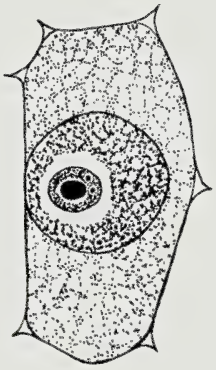

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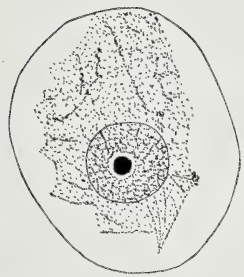

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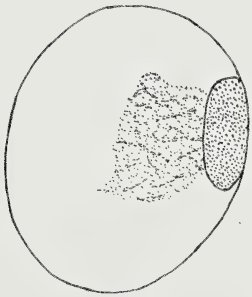

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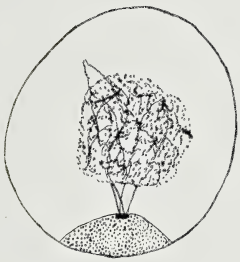

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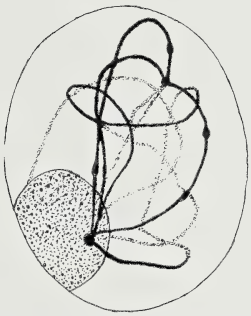

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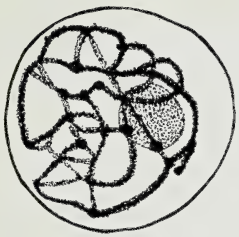

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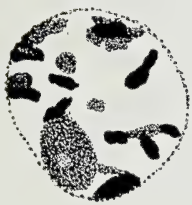

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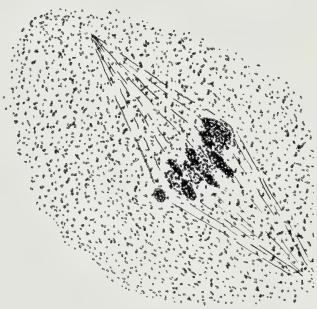

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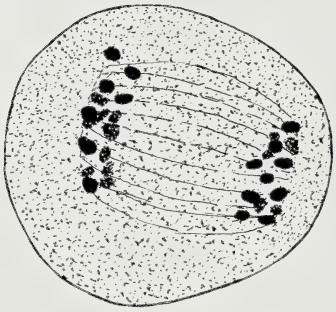

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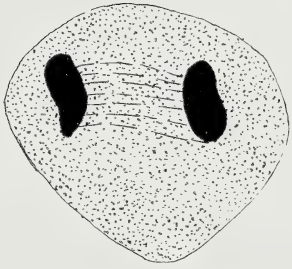

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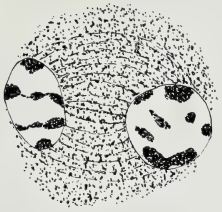

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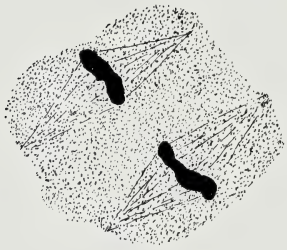

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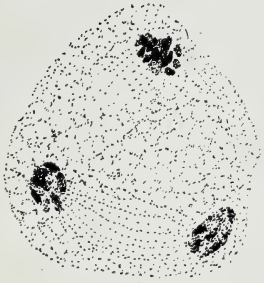

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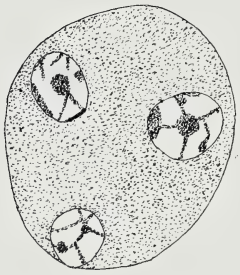

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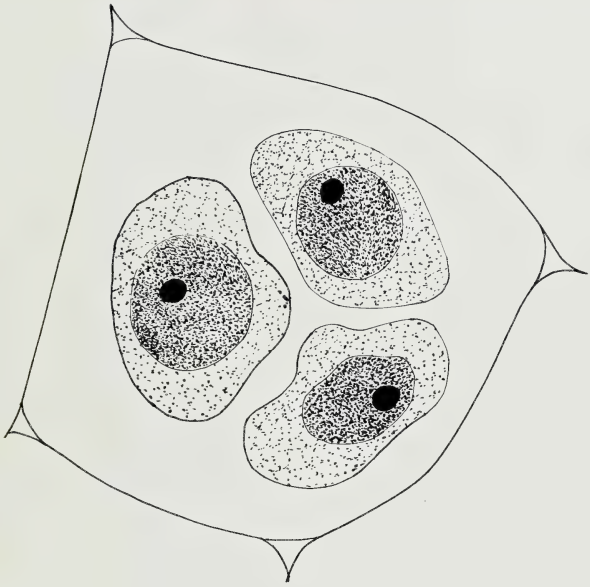

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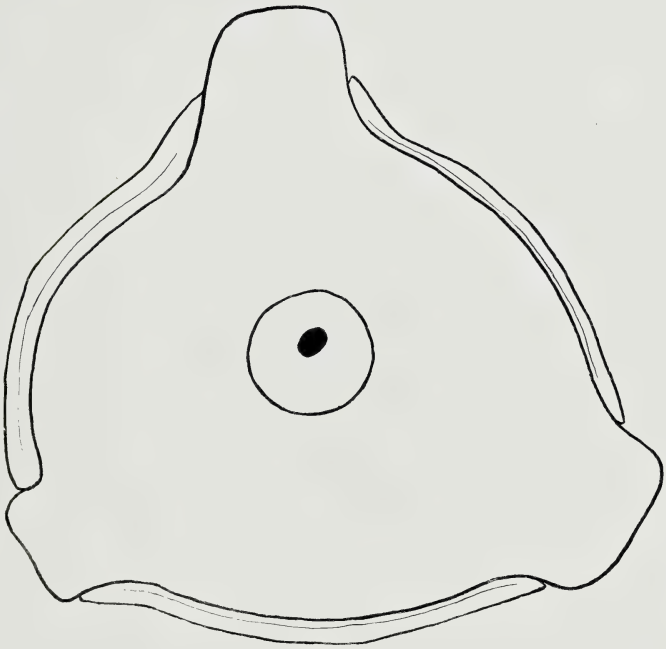

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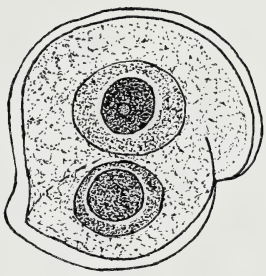

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